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Pegylation effect of chitosan based polyplex on DNA transfection

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ABSTRACT

The aim of this study was to develop hepatocyte-targeting non-viral polymeric nono-carriers for gene delivery. Chitosan was selected as the main polymer. An asialoglycoprotein receptor recognized sugar, galactose, was introduced. The methoxy poly(ethylene glycol) (mPEG) or short chain poly(ethylene glycol) diacid (PEGd) was further grafted onto galactosylated chitosan. All polyplex possessed positive charge character. The compaction of DNA by grafted chitosan was in order of chitosan-galactose-mPEG > chitosan-galactose-PEGd > chitosan-galactose where the chitosan-galactose-mPEG and pDNA formed the most stable polyplex. The polyplex prominently enhanced DNA cellular transfection as compared to naked DNA in HepG2 cells in order of chitosan-galactose/pDNA (11.6 ± 0.6–$33.0 \pm 4.4\%$) > chitosan-galactose-PEGd/pDNA (12.7 ± 2.5–$15.5 \pm 3.0\%$) > chitosan-galactose-mPEG/pDNA (9.0 ± 1.1–$12.9 \pm 2.4\%$).

1. Introduction

Chitosan is a relatively low toxic, biocompatible, and biodegradable polysaccharide with immunological, antibacterial and wound-healing activities. Several strategies have been adopted for chemical modification of chitosan through C2-amino group or C6-hydroxyl group using different substitutes (Gao et al., 2009; Gorochovceva & Makus, 2004; Laurentin & Edwards, 2003; Lin & Chen, 2007; Liu et al., 2009; Park et al., 2003; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2009). The modified chitosan is applied for drug delivery, tissue engineering, and other biomedical applications (Alves & Mano, 2008; D'Amelio et al., 2013; Muzzarelli, 2010). The free C2-amino group of chitosan is feasible to complex with negatively charged DNA as a gene delivery carrier. Poly(ethylene glycol) (PEG) is popularly used in pharmaceutics due to its hydrophilic character, high solubility, low cytotoxicity and good biocompatibility. It was reported that PEG can reduce protein opsonization of nanoparticles and subsequent phagocytosis by non-parenchymal cells of the liver *in vivo*. The shielding effect of PEG prevents nanoparticles from reticuloendothelium system (RES) uptake resulting in long-circulating characteristics (Avgoustakis, 2004; Betancourt et al., 2009; Ioele, Cione, Risoli, Genchi, & Ragno, 2005; Lu et al., 2009). The similar result has been reported by van Vlerken et al. They found that the pegylated nanoparticles avoided uptake by RES, thereby improving circulation time of nanoparticles, and the nanoparticles are retained in the tumor for prolonged period of time (van Vlerken, Duan, Little, Seiden, & Amiji, 2008). On the other hand, PEG has been used to improve solubility of chitosan in simulated gastric pH and physiological pH *via* altering molecular weight and/or substitution degree of PEG (Casettari et al., 2012; Jeong, Kim, Jang, & Nah, 2008).

Asialoglycoprotein receptor (ASGPR) receives much attention in gene targeting and also plays as a model system for studying receptor-mediated endocytosis due to its high affinity and rapid internalization rate. ASGPR is an integral membrane protein expressed on the surface of parenchymal cells of liver with high density of $1–5 \times 10^5$ receptors (Weigel & Yik, 2002). Nanocarriers (*e.g.*, nanoparticles) with surface modification are necessary for specific targeting purpose. Several sugar ligands (*e.g.*, galactose, *N*-acetylgalactosamine, mannose, lactose, fructose, *etc.*) have proved to interact with ASGPR with various extents. Galactose has been proved recognition of ASGPR through many *in vitro* and *in vivo* studies. Wang et al. (2012) used galactose and PEG modified liposome to encapsulate doxorubicin which demonstrated better targeting efficiency and achieved 94% tumor growth inhibition. Jiang et al. prepared PEG-galactose followed by grafted onto the amino group of chitosan-PEI. It had better cellular transfection than PEI after intravenous injection (Jiang et al., 2008). Chen et al. used lactobionic acid and glycyrrhetinic acid to prepare dual-ligand modified chitosan. Its transfection efficiency in ASGPR high-expressed BEL-7402 cells was higher than in ASGPR-free LO2 hepatic normal cells (Chen et al., 2012).

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Most of the studies modified chitosan through C2-NH_2 group due to simple and easy synthetic procedure. However, the positive charge of C2-NH_2 group plays an important role in complex with negatively charged DNA for gene delivery. Some studies were designed to modify chitosan through C6-OH but leave C2-NH_2 group available for DNA complexation. Jiang, Wu, Xu, Wang, and Zeng (2011) used C6-OH modified chitosan to complex with various weight ratios of DNA, and found the molar ratio of polymer/DNA 20:1 expressed the highest cellular transfection. The chemical modification of chitosan by grafting lactobionic acid, as a receptor ligand, through C6-OH position of chitosan has been demonstrated (Lin, Chen, & Liu, 2009; Lin, Chen, Liu, Chen, & Chang, 2011). Lactobionic acid is an endogenous substance present in the human body (Yu & van Scott, 2004). The chemical structure of lactobionic acid contains a galactose unit and a gluconic acid unit linked by ether linkage. The carboxyl group of gluconic acid unit reacts with the amino group of chitosan to form an amide linkage. The lactobionic acid grafted chitosan demonstrated higher transfection efficiency than ligand-free chitosan (45.3% *vs* 19.8%) in ASGPR overexpressed HepG2 cells. Zhang et al. (2009) grafted galactose onto C6-OH followed by pegylation from C2-NH_2 group. They demonstrated no cytotoxicity of modified chitosan in HEK 293 kidney cancer cells. However, it was lack of data to verify the feasible application of this modified chitosan in drug and/or gene delivery.

The present study was aimed to develop a hepatocyte-targeting non-viral polymeric nano-carrier for gene delivery. Chitosan was selected as the main polymer. In order to have specific liver targeting activity, an ASGPR recognized sugar molecule, galactose, was introduced into C6-OH of chitosan. The hydrophilic methoxy poly(ethylene glycol) (mPEG) or short chain PEG diacid (PEGd) was grafted onto galactosylated chitosan further through its C2-NH_2 position to increase solubility and stability of chitosan *in vivo*. The synthesized chitosan derivatives were characterized by FTIR, NMR and GPC, and the galactose, mPEG and PEGd graft contents were determined. The galactosylated chitosan grafted with mPEG or PEGd was applied to complex with plasmid DNA, and the performance of polymer/DNA polyplex was characterized. The ability of condensing negatively charged plasmid DNA by modified chitosan, the stability of polymer/DNA polyplex and its cellular transfection were evaluated.

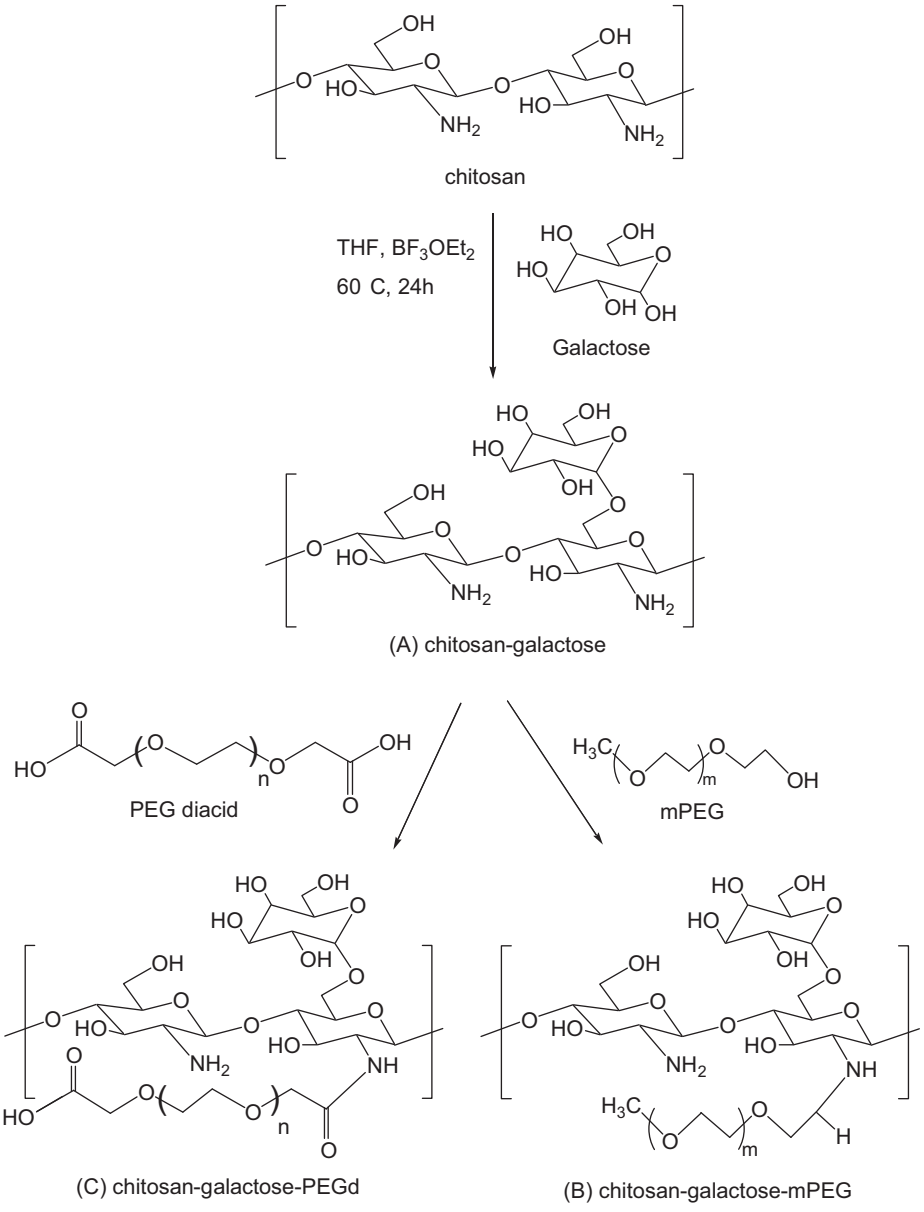

Fig. 1. Scheme for synthesis of (A) chitosan-galactose, (B) chitosan-galactose-mPEG, and (C) chitosan-galactose-PEGd.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (CS, M_w 260 kDa, M_n 72 kDa, deacetylation degree 76.3 ± 2.1%) and poly(ethylene glycol) diacid (PEGd, M_n 600 Da) were from Aldrich Chemical Company, Inc., (WI, USA). Methoxy poly(ethylene glycol) (mPEG, *MW* 5,000 Da), boron trifluoride diethyl ether (BF_3•OEt_2), and anthrone were from Fluka Chemical Company Inc. (Buchs, Switzerland). D(+)-Galactose (99 + %) and *N*-hydroxysuccinimide (NHS) were from Acros Organics Co. Inc. (Geel, Belgium). Sodium nitrite ($NaNO_2$) was from Showa Chemical Co. Ltd. (Tokyo, Japan). Sodium cyanoborohydride ($NaCNBH_3$, 95%) was from Alfa Aesara Johnson Matthey Co. Inc. (Massachusetts, USA). 1-Ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride (EDC) was from TCI Chemical Industry Co. Ltd. (Tokyo, Japan). Minimum essential media (MEM) was from Biological Industries Israel Beit-Haemek Ltd. (Beit HaEmek, Israel). Plasmid encoding enhanced green fluorescent protein (*p*EGFP-N1, 4.7 kb) was kindly provided by Professor Jiin Long Chen from National Defense Medical Center in Taiwan. The HepG2 cancer cell line was a gift from Dr. Hui-Lin Wu in Hepatitis Research Center of National Taiwan University Hospital in Taiwan.

2.2. Synthesis of chitosan-based polymers

Fig. 1(A) shows the procedures to synthesize chitosan-galactose (Chitosan(1)) (Lin et al., 2009, 2011). Chitosan was deacetylated in NaOH aqueous solution (50%w/v) at 140 °C for 4 h followed by depolymerized in 0.1 M sodium nitrite acetic solution at room temperature for 3 h. The obtained deacetylated depolymerized chitosan (DADPCS) was reacted with galactose at feed molar ratio 1:2.5 in the mixture of tetrahydrofuran (THF) and boron trifluoride diethyl etherate (BF_3•OEt_2) at 60 °C under N_2 for 24 h. The solvent was removed by rotary evaporation, and the mixture was dialyzed (*MW cut-off 500-1000 Dalton*) followed by freeze dried.

Fig. 1(B) shows the procedures to synthesize chitosan-galactose-mPEG (Chitosan(2)). mPEG was dissolved in a mixture of DMSO and chloroform (10:1, v/v) followed by reacting with acetic anhydride at room temperature for 9 h. The ether was added to precipitate the product mPEG-CHO which was collected after filtration. The mPEG-CHO was dialyzed (*MW cut-off 500-1*,000 Da) and freeze dried. Chitosan(1) was previously dissolved in a mixture of 2% acetic acid and methanol (1:1 v/v). mPEG-CHO in deionized water was slowly added into and reacted at room temperature for 3 h followed by adding $NaCNBH_3$ aqueous solution under N_2 for further 18 h. The feed molar ratio of Chitosan(1): mPEG-CHO: $NaCNBH_3$ was 1.0:0.6:4.5. The reaction solution was concentrated by rotary evaporation, and the mixture was dialyzed (MW cut-off 6000-8000 Da) followed by vacuum dried.

Fig. 1(C) shows the procedures to synthesize chitosan-galactose-PEGd (Chitosan(3)). Chitosan(1) was previously dissolved in 1% acetic acid solution. PEGd, NHS and EDC were slowly added into and reacted at room temperature for 24 h. The feed molar ratio of Chitosan(1):PEGd:NHS:EDC was 1:7:7:7. The reaction solution was dialyzed (MW cut-off 6000-8000 Da) followed by freeze dried. The obtained Chitosan(3) was washed by acetone for three times followed by vacuum dried.

2.3. Characterization of chitosan-based polymers

The obtained Chitosan(1), Chitosan(2), and Chitosan(3) were characterized by FTIR and ^{1}H NMR, the molecular weights were analyzed by GPC. The galactosylation ratio in terms of weight percentage (W_g%) was calculated according to Eq. (1). The galactose degree of substitution (DS_g%) of Chitosan(1), Chitosan(2), and Chitosan(3) were determined by anthrone–sulfuric acid colorimetric assay (Laurentin & Edwards, 2003) and calculated according to Eqs. (2), (3), and (4), respectively.

$$\text{Galactose } W_g(\%) = \frac{\text{galactose weight in the sample}}{\text{sample weight}} \times 100\% \quad (1)$$

$$\text{galactose } DS_{g1}(\%) = \frac{(\text{galactose weight}) / \left(M_{w\ \text{galactose}}\right)}{(\text{sample weight} - \text{galactose weight}) / (M_{w\ \text{DADPCS monomer}})} \times 100\% \quad (2)$$

$$\text{galactose } DS_{g2}(\%) = \frac{(\text{galactose weight}) / \left(M_{w\ \text{galactose}}\right)}{(\text{sample weight} - \text{galactose weight} - \text{mPEG weight}) / (M_{w\ \text{DADPCS monomer}})} \times 100\% \quad (3)$$

$$\text{galactose } DS_{g3}(\%) = \frac{(\text{galactose weight}) / \left(M_{w\ \text{galactose}}\right)}{(\text{sample weight} - \text{galactose weight} - \text{PEGd weight}) / (M_{w\ \text{DADPCS monomer}})} \times 100\% \quad (4)$$

The mPEG degree of substitution (DS_{mPEG}%) of Chitosan(2) was calculated by Eq. (5) based on ^{1}H NMR data, and the pegylation weight percentage (W_{mPEG}%) was calculated by Eq. (6).

$$DS_{mPEG}(\%) = \frac{(\text{area of peak } c)_{3.5\ \text{ppm}}/3}{(\text{area of peak } d)_{3.2\ \text{ppm}}} \times 100\% \quad (5)$$

$$W_{mPEG}(\%) = \frac{DS_{mPEG} \times M_{w\ mPEG}}{(DS_{mPEG} \times M_{w\ mPEG}) + (100\% \times M_{w\ monomer}) + \left(DS_{g2} \times M_{w\ galactose}\right)} \times 100\% \quad (6)$$

Similarly, the PEGd degree of substitution (DS_{PEGd}%) and the pegylation weight percentage (W_{PEGd}%) of Chitosan(3) were calculated by Eqs. (7) and (8), respectively.

$$DS_{PEGd}(\%) = \frac{(\text{area of peak } c')_{4.15\text{ppm}}/2}{(\text{area of peak } d)_{3.1\text{ppm}}} \times 100\% \quad (7)$$

$$W_{PEGd}(\%) = \frac{DS_{PEGd} \times M_{w\ PEGd}}{(DS_{PEGd} \times M_{w\ PEGd}) + (100\% \times M_{w\ DADPCS\ monomer}) + \left(DS_{g3} \times M_{w\ galactose}\right)} \times 100\% \quad (8)$$

2.4. Gel permeation chromatography (GPC)

The molecular weight as well as molecular weight distribution in terms of polydispersity of modified chitosan was determined by gel permeation chromatography (GPC) equipped with a refractive index detector (Shimadzu RID-10A, Japan). Two linear columns (Ultrahydrogel™ 500 and DP 120, 7.8 × 300 mm, Waters) were applied and acetate buffered solution at pH 5.0 was used as the eluting solvent at a flow rate of 0.8 mL/min at 35 °C. The calibration curve was constructed using different molecular weights of poly(ethylene glycol) standards. The molecular weight of modified chitosan was re-calculated from the calibration curve based on the measured retention time.

2.5. Galactose determination

The content of galactose grafted onto chitosan was measured by colorimetric assay using anthrone sulfuric acid (Laurentin & Edwards, 2003). Several known concentrations of galactose solutions were placed in a 96-well pre-cooled at 4 °C. The fresh prepared anthrone–sulfuric acid in an ice bath was added into the 96-well. The 96-well was heated at 90 °C for 6 min followed by cooled to room temperature. The absorbance was determined by spectrophotometer at 630 nm. The calibration curve was constructed based on several concentrations of galactose and their absorbance. The polymer samples were prepared according to the same procedure, and the corresponding concentration was re-calculated from the calibration curve based on the measured absorbance.

2.6. Cytotoxicity of galactosylated and pegylated chitosan

The cytotoxicity of Chitosan(1), Chitosan(2), and Chitosan(3) was investigated. HepG2 cells were cultured in the modified Eagle's medium containing 10% fetal bovine serum, sodium bicarbonate, nonessential amino acids and sodium pyruvate. The cells were seeded in a 96-well plate at a density of 9000 cells per well and maintained in a humidified incubator at 37 °C in 5% CO_2 for 24 h. Serial dilutions of polymer solution in cultured medium were added into each well and incubated at 37 °C for 24 h. The cultured medium without polymer solution was the control. The medium was removed, and the MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium was added and incubated at 37 °C for 4 h (Ciapetti, Cenni, Pratelli, & Pizzoferrato, 1993). The resulting formazan was solubilized in dimethyl sulfoxide, and the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Power Wave XS, BioTek, Winooski, VT) at 570 nm (Liu & Lin, 2013).

2.7. Preparation of polymer/pDNA polyplex

Chitosan(1), Chitosan(2), and Chitosan(3) were complexed with negatively charged pDNA at various weight ratios of 2:1, 10:1 and 20:1. Each polymer was previously dissolved in 1% acetic solution. The plasmid DNA was dissolved in sterile distilled water followed by slowly added into polymer solution and stirred for 3 min. The resulting polyplex was stood for 3 h at room temperature. The polyplex solution was centrifuged at 16,000 × *g* for 30 min. The supernatant was removed, and the distilled water was added to re-disperse the polyplex. The particle size and zeta potential were measured by using Zetasizer nano analyzer (Nano-ZS 90, Malvern Instruments Ltd., Worcestershire, UK) at 25 °C. The morphology of polymer/pDNA polyplex was observed by transmission electron microscope (Philips Tecnai F30, Philips, Netherlands). The stability of polyplex was evaluated their particle size change during storage at 4 °C for 28 days.

2.8. Transfection of polymer/pDNA polyplex

The transfection of Chitosan(1)/pDNA, Chitosan(2)/pDNA, and Chitosan(3)/pDNA polyplex was evaluated in ASGPR overexpressed HepG2 cancer cells. The HepG2 cancer cells were seeded in a 6-well plate at a density of 6×10^5 cells/well and incubated at 37 °C for 24 h. After that the medium was removed, the serum-free MEM medium containing polymer/pDNA polyplex or naked pDNA was added into each well and incubated for 24 h. The phosphate-buffered solution (PBS) was added after the medium was removed. The cell suspension was centrifuged at 1200 rpm for 5 min. The cells were collected and resuspended in pH 7.4 PBS for flow cytometric analysis in the fluorescence channel FL-1 at an excitation wavelength 488 nm and an emission wavelength 530 nm. A total of 10,000 cells were analyzed for each sample, and the upper limit of background fluorescence was set no more than 1%. Data were presented as mean ± standard deviation. Comparison between two groups was analyzed by Student's *t*-test, and the difference was considered significant at $p < 0.05$ or 0.01.

Table 1
The deacetylation degree, molecular weight, galactosylation and pegylation of chitosan-galactose, chitosan-galactose-mPEG, and chitosan-galactose-PEGd.

	Chitosan-galactose	Chitosan-galactose-mPEG	Chitosan-galactose-PEGd
DD (%)	–	–	–
M_w (Da)	6200	8500	7600
M_n (Da)	4000	5500	5200
PD	1.56	1.54	1.46
W_g (%)	16.7	13.2	13.8
DS_g (%)	18.4	27.3	28.2
W_{PEG} (%)	–	42.6	52.8
DS_{PEG} (%)	–	3.7	43.5

3. Results and discussion

3.1. Characterization of chitosan-galactose (Chitosan(1))

Fig. 2(C) shows the ^{1}H NMR spectrum of Chitosan(1). The MW and galactosylation data of Chitosan(1) are listed in Table 1. The M_w, M_n and polydispersity of Chitosan(1) were 6200 Da, 4000 Da, and 1.56, respectively. The corresponding galactose grafting weight percentage (W_g(%)) and degree of substitution (DS_{g1}(%)) were 16.7% and 18.4%, respectively.

3.2. Characterization of chitosan-galactose-mPEG (Chitosan(2))

PEG plays an important role in preventing nanoparticles aggregation and avoiding nanoparticles eliminated by RES. The galactosylated chitosan was further pegylated by mPEG, and the relevant characterization data of Chitosan(2) are summarized in Table 1. The corresponding M_w, M_n and polydispersity of Chitosan(2) were 8500 Da, 5500 Da, and 1.54, respectively. Fig. 2(A) shows the ^{1}H NMR spectrum of Chitosan(2). The peaks *a* at 3.6–4.0 ppm were assigned to C3–C6 protons of chitosan and the protons of galactose, and peak *d* at 3.2 ppm was assigned to C2–H of chitosan. The peak *b* at 3.6–3.8 ppm was assigned to the protons of mPEG repeat units (—CH_2—CH_2—O—), and peak *c* at 3.5 ppm was assigned to —OCH_3 of mPEG. The W_g(%) and DS_{g2}(%) of galactose calculated by Eqs. (1) and (3) were 13.2% and 27.3%, respectively. The mPEG grafting weight percentage (W_{mPEG}%) calculated by Eq. (5) was 42.6%, and the corresponding degree of substitution, DS_{mPEG}%, calculated by Eq. (4) was 3.7% based on the integration area of peak *c* and peak *d* in ^{1}H NMR spectrum. The degrees of substitution of galactose and mPEG of mPEGylated-galactosylated-chitosan developed by Zhang et al. (2009) were 0.09% and 0.3%, respectively, which were much lower than ours. It seemed that the current method applied to graft galactose and mPEG onto chitosan was more efficient in terms of higher grafting values than theirs.

3.3. Characterization of chitosan-galactose-PEGd (Chitosan(3))

Another approach was designed to pegylate Chitosan(1) with short chain PEG diacid (MW 600 Da), and the relevant characterization data of Chitosan(3) are summarized in Table 1. The corresponding M_w, M_n and polydispersity of Chitosan(3) were 7600 Da, 5200 Da, and 1.46, respectively. Fig. 2(B) shows the ^{1}H NMR spectrum of Chitosan(3). The peak *c′* at 4.15 ppm and peak *b* at 3.6 ppm were assigned to the C—H next to —COOH of PEG diacid and the repeat units (—CH_2—CH_2—O—) of PEG diacid. The peaks

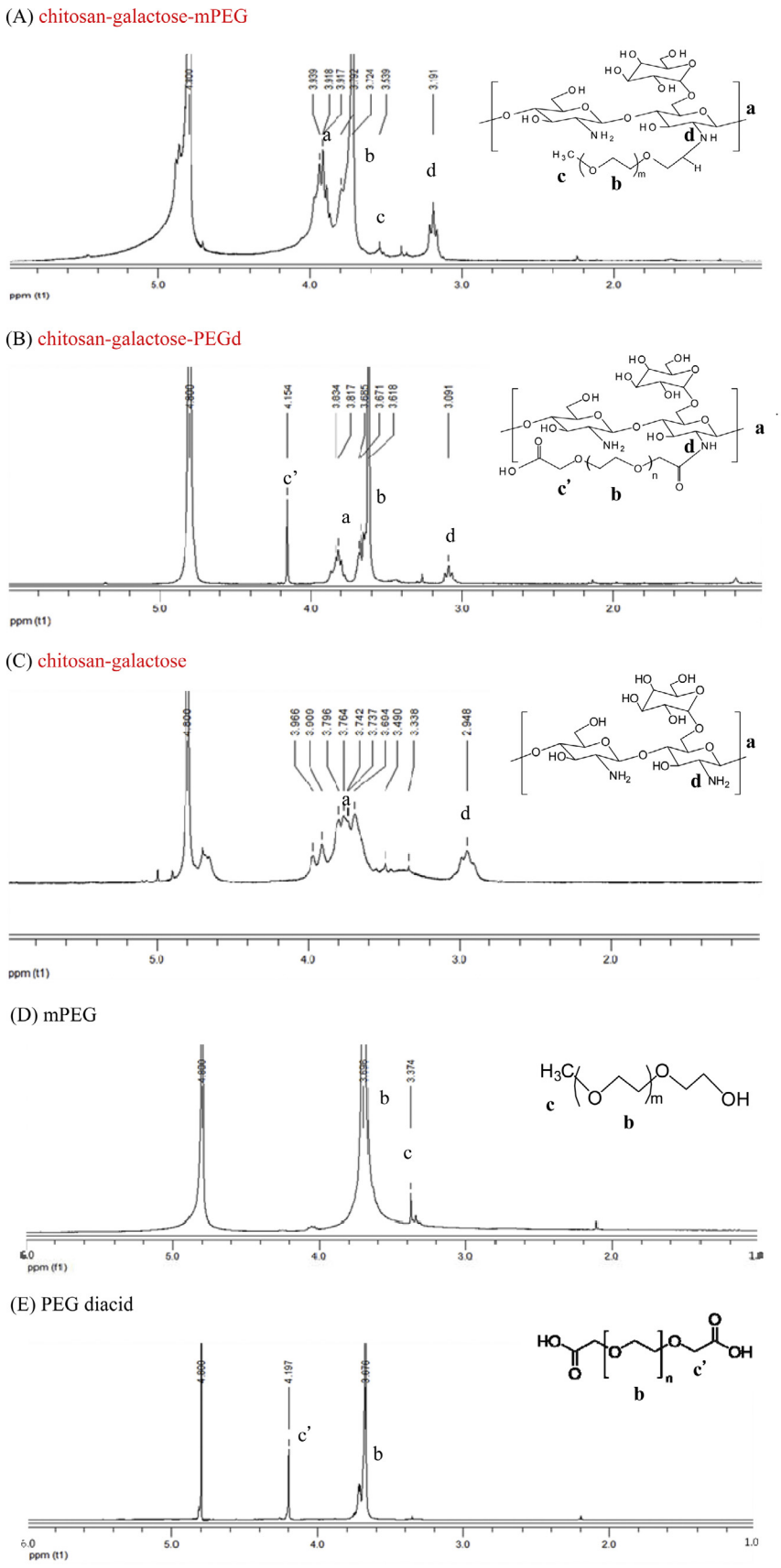

Fig. 2. The ^{1}H NMR spectra of (A) chitosan-galactose-mPEG, (B) chitosan-galactose-PEGd, (C) chitosan-galactose, (D) mPEG, and (E) PEG diacid.

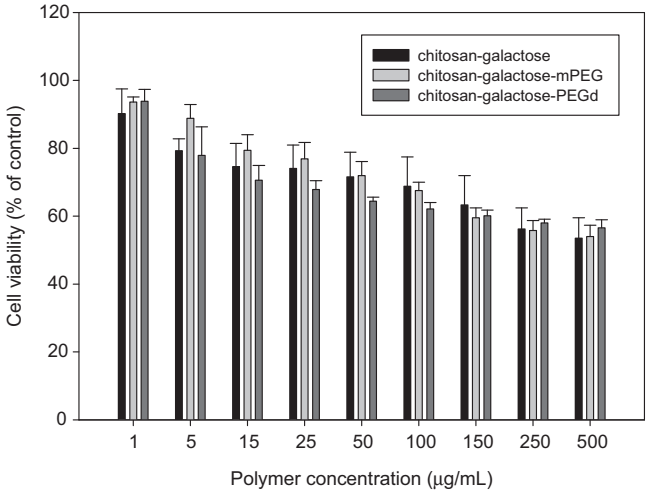

Fig. 3. The cellular viability of chitosan-galactose, chitosan-galactose-mPEG, and chitosan-galactose-PEGd. The values represent mean ± SD, $n = 3$.

a at 3.7–3.9 ppm was assigned to C3–C6 protons of chitosan and the protons of galactose, and peak *d* at 3.1 ppm was assigned to C2–H of chitosan. The galactose grafting $W_g(\%)$ and $DS_{g3}(\%)$ were 13.8% and 28.2%, respectively. The weight percentage of pegylation ($W_{PEGd}(\%)$) was 52.8%, and the corresponding $DS_{PEGd}(\%)$ was 43.5% based on the integration area of peak *c*′ and peak *d* in ^{1}H NMR spectrum.

3.4. Cytotoxicity of galactosylated and pegylated chitosan

Fig. 3 illustrates the cellular viability of Chitosan(1), Chitosan(2), and Chitosan(3) in HepG2 cells. The cytotoxicity of grafted chitosan was similar irrespective of the presence of PEG and PEG chain length, and there were at least 80% cells viable at polymer concentration ≤5 μg/mL. All of the grafted chitosan had IC_{50} corresponding to 50% cytotoxicity higher than 500 μg/mL. It indicated that the galactosylated-pegylated-chitosan had low cytotoxicity and was much safe being used *in vivo*. Kim, Shin, and Lee (1999) reported that the cytotoxicity of PEG with molecular weight greater than 3000 Da was ignorable. Similarly, Mao et al. (2005) found that the low cytotoxicity of PEG-conjugated-chitosan was observed in PEG M_w 5000 Da rather than 550 Da. Nevertheless, there was no difference in cytotoxicity between mPEG (5000 Da) and short chain PEG diacid (600 Da) grafted chitosan in our current study.

3.5. Characterization of polymer/DNA polyplex

The galactosylated-pegylated-chitosan was applied as a DNA delivery carrier. Complex of cationic chitosan and negatively charged plasmid DNA spontaneously formed polyplex due to electrostatic interaction. Fig. 4(A) illustrates the particle size of Chitosan(1)/pDNA, Chitosan(2)/pDNA, and Chitosan(3)/pDNA with various polymer/DNA weight ratios. The particle size of Chitosan(2)/pDNA polyplex with polymer/pDNA weight ratio 2:1, 10:1, and 20:1 was 159.9 ± 43.0, 104.6 ± 8.1, and 98.7 ± 6.6 nm, respectively. The compaction of DNA by Chitosan(2) was prominent when polymer/DNA weight ratio was increased from 2:1 to 10:1 where the particle size was significantly decreased. Further increase in polymer/DNA weight ratio to 20:1 did not change particle size too much. The sterically repulsive nature of mPEG protected Chitosan(2)/pDNA from secondary aggregation and formed polyplex with reliable particle size in the range of 100–200 nm. The similar phenomenon has been reported by

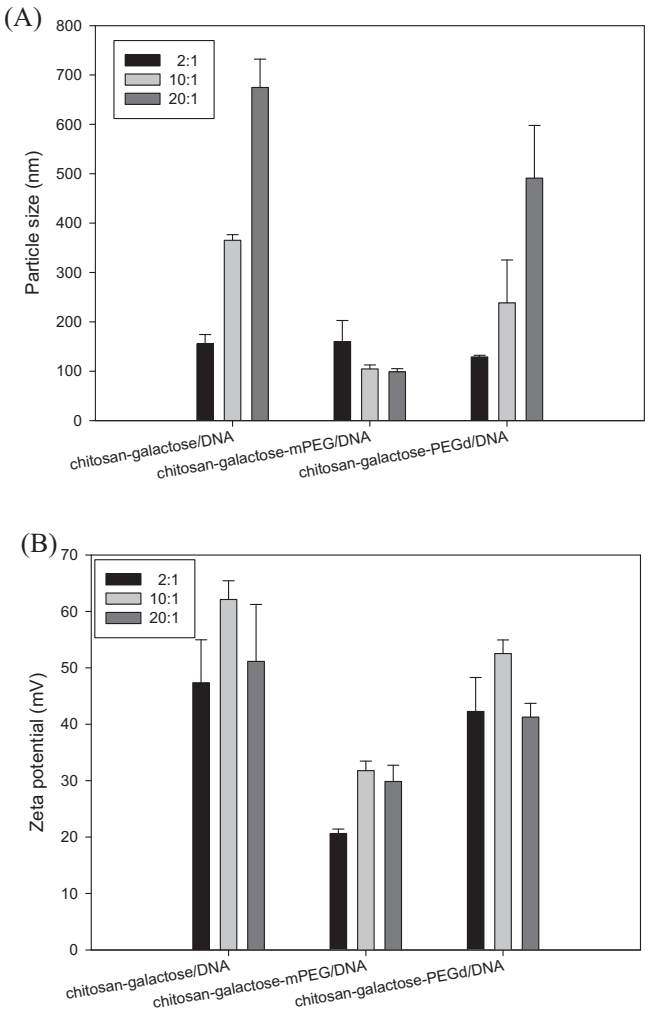

Fig. 4. The (A) particle size (nm) and (B) zeta potential (mV) of chitosan-galactose/pDNA, chitosan-galactose-mPEG/pDNA, and chitosan-galactose-PEGd/pDNA polyplex with polymer/DNA weight ratios 2:1, 10:1 and 20:1. The values represent mean ± SD, $n = 3$.

Kataoka, Harada, and Nagasak (2001) where the polyionic PEG-poly(L-lysine) block copolymer was complexed with positively charged pDNA. They mentioned that the PEG corona surrounded on micelle surface decreased the local dielectric constant which facilitated DNA compacted by PEG–PLys. However, only the 2:1(w/w) polyplex of Chitosan(3)/pDNA and Chitosan(1)/pDNA had particle size less than 200 nm. The increase of polymer (*e.g.*, polymer/DNA 10:1 and 20:1) was fail to sufficiently compact DNA into polyplex of Chitosan(3) and Chitosan(1) which resulted in quite large in particle size. The lack of steric protection by these two polymers accounted for resulting polyplex with quite large size. All of these results implied that the compaction of DNA by grafted chitosan was in order of Chitosan(2)/pDNA > Chitosan(3)/pDNA > Chitosan(1)/pDNA, and the best DNA compaction was achieved by Chitosan(2). The morphology of Chitosan(2)/pDNA polyplex is illustrated in Fig. 5.

Fig. 4(B) illustrates the zeta potential of polyplex with various polymer/DNA weight ratios. All polyplex possessed positive charge character in order of Chitosan(2)/pDNA (+20–30 mV) < Chitosan(3)/pDNA (+40–50 mV) < Chitosan(1)/pDNA (+45–60 mV). The mPEG polymer chains surrounded on the polyplex surface diminished the positive charge of chitosan resulting in the lowest zeta potential of Chitosan(2)/pDNA polyplex. The chain length of mPEG polymer was longer than PEG diacid where mPEG formed better surface

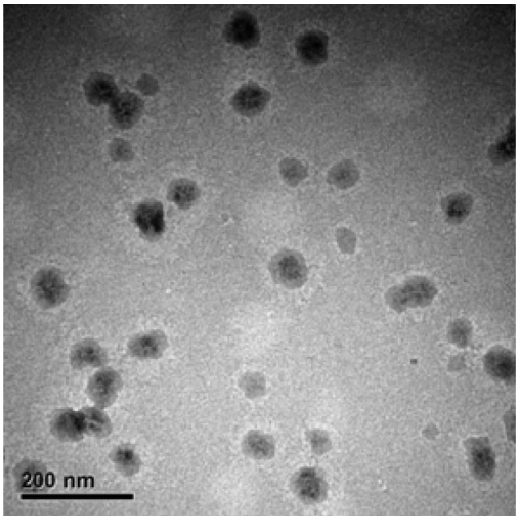

Fig. 5. The TEM image of chitosan-galactose-mPEG/pDNA polyplex with polymer/DNA weight ratio 20:1.

coverage on Chitosan(2)/pDNA polyplex. On the other hand, the shorter chain length of PEG diacid exerted less surface coverage than mPEG and resulted in the zeta potential of Chitosan(3)/pDNA higher than Chitosan(2)/pDNA but less than Chitosan(1)/pDNA.

3.6. Stability of polyplex

Fig. 6 illustrates the stability in terms of percentage of particle size change of polyplex after storage at 4 °C for 28 days. Most of Chitosan(2)/pDNA and Chitosan(3)/pDNA polyplex maintained their particle size at the end of 28 days except 20:1(w/w) Chitosan(3)/pDNA polyplex. It lost stability after storage for 21 days where the polyplex was aggregated in terms of enlarging particle size much. All of these results indicated that Chitosan(2) was not only capable of condensing plasmid DNA but also formed stable polyplex as compared to Chitosan(1) and Chitosan(3). The presence of mPEG of Chitosan(2) played an important role in preventing polyplex aggregation and maintaining its stable nature where the hydrophilic PEG chains surrounded on the outer shell of the polyplex and extended in the aqueous environment to exert shielding effect (Betancourt et al., 2009; Lin et al., 2009; Lu et al., 2009).

3.7. Transfection of polyplex

Fig. 7 illustrates the transfection efficiency of polyplex in asialoglycoprotein receptor (ASGPR) overexpressed HepG2 cells. The transfection of naked plasmid DNA (*p*EGFP-N1) was similar to the negative control (MEM medium only). However, all of the polyplex enhanced pDNA cellular transfection as compared to naked DNA in order of Chitosan(1)/pDNA > Chitosan(3)/pDNA > Chitosan(2)/pDNA. Increase in polymer/DNA weight ratios of Chitosan(1)/pDNA polyplex from 2:1 to 20:1 prominently increased transfection efficiency in terms of producing more green fluorescent proteins in ASGPR overexpressed HepG2 cells. This provided the evidence to ensure the specific targeting of galactose to ASGP receptor. The galactose grafting weight percentage (W_g%) of Chitosan(1), Chitosan(2) and Chitosan(3) were 16.7, 13.2 and 13.8%, respectively. Although the grafted galactose of Chitosan(1) was similar to the other two kinds of galactosylated-pegylated-chitosan, its galactose moiety was fully exposed and specifically bound to ASGP receptor to enhance cellular transfection the most. Nevertheless, the shielding effect

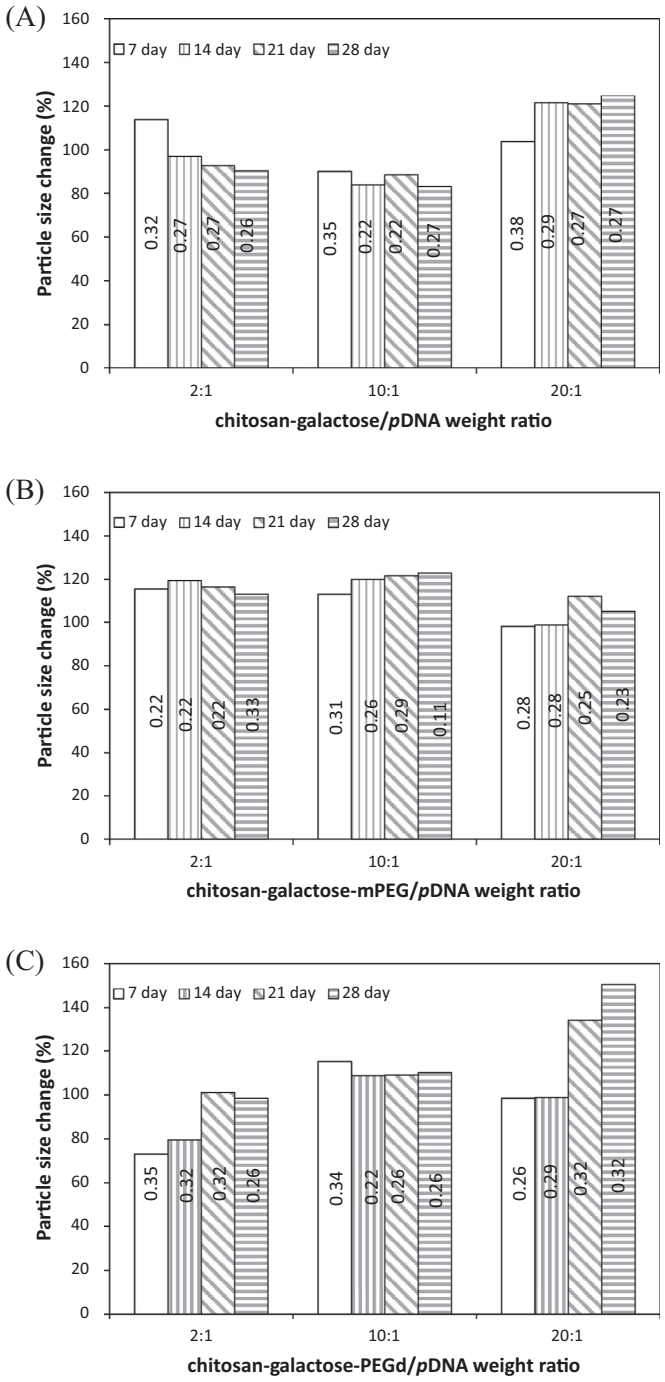

Fig. 6. The stability in terms of particle size change (%) of (A) chitosan-galactose/pDNA, (B) chitosan-galactose-mPEG/pDNA, and (C) chitosan-galactose-PEGd/pDNA polyplex during storage at 4 °C for 28 days. The value in each column indicates the polydispersity index (PDI).

of mPEG on the surface of Chitosan(2)/pDNA polyplex diminished the specific targeting ability of galactose to ASGP receptor resulting in the lowest cellular transfection in HepG2 cells as compared to the other polyplex. On the other hand, the Chitosan(3)/pDNA polyplex was covered by short chain PEG diacid. The shielding effect of PEG diacid was not so prominent as mPEG which accounted for the cellular transfection of Chitosan(3)/pDNA polyplex higher than Chitosan(2)/pDNA but lower than Chitosan(1)/pDNA.

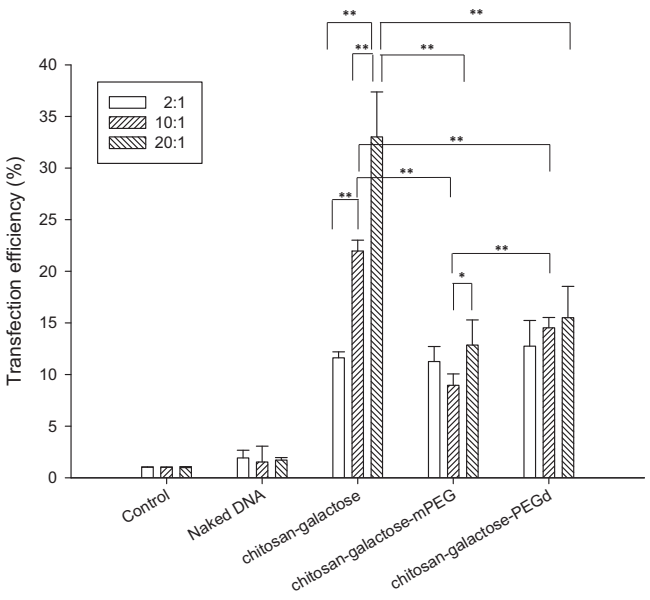

Fig. 7. Transfection of (−)control, naked plasmid DNA, chitosan-galactose/pDNA, chitosan-galactose-mPEG/pDNA, and chitosan-galactose-PEGd/pDNA polyplex in asialoglycoprotein overexpressed HepG2 cells for 24 h. The values represent mean ± SD, $n = 3$. * $p < 0.05$ and ** $p < 0.01$ by Student's t-test.

4. Conclusion

The galactosylated-pegylated-chitosan with asialoglycoprotein receptor targeting ability was developed for gene delivery. The chitosan was chemically grafted by galactose and different chain lengths of hydrophilic methoxy poly(ethylene glycol) or poly(ethylene glycol) diacid. The concentration of grafted chitosan corresponding to 50% cytotoxicity was higher than 500 μg/mL. The positively charged grafted chitosan formed polyplex with negatively charged plasmid DNA, and the compaction of DNA by grafted chitosan was in order of Chitosan(2)/pDNA > Chitosan(3)/pDNA > Chitosan(1)/pDNA. All polyplex enhanced DNA cellular transfection as compared to naked DNA. Although Chitosan(2)/pDNA polyplex maintained its particle size for longest time, the shielding effect of methoxy poly(ethylene glycol) diminished the specific targeting ability of galactose to asialoglycoprotein receptor resulting in the lowest cellular transfection in HepG2 cells. Through this study elucidated the role of poly(ethylene glycol) in chitosan-based polyplex stability and cellular transfection.

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